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Certification

On November 24, 1999, Dr. Roland Kreutzer of Weidenberg, Germany and Dr. Stefan Limmer of Bayreuth, Germany, filed a patent application with the title

"Method and Medication for Inhibiting the Expression of a Defined Gene"

by submitting it to the German Patent and Trademark Office and declaring that they are claiming an internal priority of the patent application in the Federal Republic of Germany of January 30, 1999, no. 199 03 713.2.

The attached document is a true and accurate reproduction of the original documents of this patent application.

German Patent and Trademark Office has assigned this patent application a preliminary classification of C 12 N and A 61 K of the International Patent Classification.

Munich, May 11, 2000

German Patent and Trademark Office

President

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Application No.: 199 56 568.6

Abstract

This invention relates to a medication having at least one oligoribonucleotide (dsRNA) having a double-stranded structure for inhibiting the expression of a defined gene, whereby one strand of the dsRNS is complementary with this gene in at least some regions.

Method and Medication for Inhibiting Expression of a Defined Gene

This invention relates to a method according to the preamble of Claim 1. It also relates to a medication and a use of double-stranded oligoribonucleotides.

Such a method is known from International Patent WO 99/32619, which was published subsequently. The known method is directed at inhibiting expression of genes in cells of invertebrates. To do so, it is necessary for the double-stranded oligoribonucleotide to have a sequence with a length of at least 50 bases identical to that of the target gene. To achieve efficient inhibition, the identical sequence must have a length of 300 to 1000 base pairs. The synthesis complexity in producing such an oligoribonucleotide is high.

German Patent 196 31 919 C2 describes an anti-sense RNA with special secondary structures, the anti-sense RNA being in the form of a vector coding for it. The anti-sense RNA is an RNA molecule that is complementary to regions of the mRNA. Inhibition of gene expression is achieved by binding to these regions. This inhibition may be used in particular for diagnosis and/or treatment of diseases, e.g., tumor diseases or viral infections. It is a disadvantage that the anti-sense RNA must be introduced into the cell in an amount at least equal to the amount of mRNA. The efficacy of the known anti-sense method is not especially high.

US Patent 5,712,257 describes a medication containing mismatched double-stranded RNA (dsRNA) and biologically active mismatched fragments of dsRNA in the form of a ternary complex with a surfactant. The dsRNA used here consists of synthetically produced nucleic acid single strands without a defined base sequence. The single strands enter into irregular base pairings, so-called "non-Watson-Crick" base pairings, so that mismatched double strands are formed. The known dsRNA is used to inhibit the replication of retroviruses such as HIV. The replication of the retrovirus can be inhibited if non-sequence-specific dsRNA is introduced into the cells. This results in induction of interferon, so that virus replication is inhibited. The inhibiting effect, i.e., the efficacy of this method is low.

It is known from A. Fire et al., Nature, vol. 391, pp. 806 that dsRNA, one of whose strands is complementary in some sections with a gene of a nematode to be inhibited, will inhibit the expression of this gene with a high efficacy. The theory is that the particular efficacy of the dsRNA used in cells of the nematode is not based on the anti-sense principle, but instead might be due to catalytic properties of the dsRNA and/or to enzymes induced by dsRNA. – This article does not

make any statements about the efficacy of specific dsRNA with respect to inhibition of gene expression, in particular in mammalian cells.

The object of the present invention is to eliminate the disadvantages of the related art. In particular, this is to provide the most effective possible method and medication and the most efficient possible use for production of a medication with which an especially effective inhibition of expression of a defined gene can be achieved.

This object is achieved by the features of Claims 1, 2, 33, 34 and 66 and 67. Advantageous embodiments are derived from Claims 3 through 32, 35 through 65 and 68 through 100.

According to a feature of this invention pertaining to the process, the region that is complementary to the target gene has at most 49 successive nucleotide pairs.

The oligoribonucleotides according to this invention are those having a defined nucleotide sequence in at least some sections. The defined section may be limited to the complementary region. However, it is also possible for the double-stranded oligoribonucleotide to have a defined nucleotide sequence on the whole.

It has surprisingly been found that even with a maximum length of 49 base pairs in the complementary region, effective inhibition of expression of the target gene can be achieved. Corresponding oligoribonucleotides can be prepared at a lower production cost.

Especially dsRNA with a length of more than 50 nucleotide pairs induces certain cellular mechanisms in mammalian cells and human cells, e.g., dsRNA-dependent protein kinase or the 2-5A system. This leads to disappearance of the interference effect mediated by the dsRNA having a defined sequence. This blocks protein biosynthesis in the cell. This disadvantage in particular is eliminated by the present invention.

In addition, this greatly facilitates the incorporation of dsRNA with a short chain length into the cells or the cell nucleus in comparison with dsRNA having a longer chain length.

If dsRNA is used as the active ingredient, it has proven advantageous for the dsRNA to be packaged in micellar structures, preferably in liposomes. The dsRNA may likewise be enclosed in natural viral capsids or in synthetic capsids produced by chemical or enzymatic methods or in structures derived therefrom. The features mentioned above make it possible to incorporate the dsRNA into defined target cells.

According to another feature of this embodiment, the dsRNA has 10 to 1000, preferably 15 to 49 base pairs. The dsRNA may thus be longer than the complementary region to the target gene. The complementary region may be arranged in terminal position or incorporated into the dsRNA. Such a dsRNA or a vector provided for coding for same may be produced synthetically or enzymatically by conventional methods.

The gene to be inhibited is expediently expressed in eukaryotic cells. The target gene may be selected from the following group: oncogene, cytokin gene, Id protein gene, development gene, prion gene. It may also be expressed in pathogenic organisms, preferably in Plasmodia. It may a component of a virus or viroid, preferably one that is a human pathogen. The proposed medication makes it possible to treat genetically controlled disease such as cancer, viral disease or Alzheimer's disease.

The virus or viroid may also be one that is pathogenic for plants or animals. In this case, the medication according to this invention also makes it possible to treat veterinary or horticultural diseases.

According to another feature of this embodiment, the dsRNA is designed to be double-stranded in some sections. The complementary region is formed from two separate RNA single strands or from self-complementary regions of a preferably circular, topologically closed RNA single strand.

The ends of the dsRNA may be modified to counteract degradation in the cell or dissociation into single strands. Dissociation occurs in particular when using low concentrations or short chain length. For especially effective inhibition of dissociation, the cohesion of the complementary region induced by the nucleotide pairs may be increased by at least one, preferably two additional chemical linkages. A dsRNA according to this invention whose dissociation is reduced has a greater stability with respect to enzymatic and chemical degradation in the cell and in the body.

In particular when using a vector according to this invention, the complementary region may be formed from self-complementary regions of a RNA hairpin loop. The nucleotides are chemically modified in the area of the loop between the double-stranded structure to protect them from degradation.

The chemical linkage is expediently formed by a covalent or ionic bond, a hydrogen bridge bond, hydrophobic interactions, preferably van der Waals interactions or stacking interactions or by metal ion coordination. According to an especially advantageous feature of this embodiment. This chemical linkage may be produced on at least one end, preferably on both ends of the complementary region.

It has also proven advantageous that the chemical linkage is formed by at least one or more groups of compounds, these groups of compounds preferably being poly(oxyphosphinicooxy-1,3-propanediol) chains and/or polyethylene glycol chains. The chemical linkage may also be formed by purine analogs used instead of purines in the complementary region. It is also advantageous that the chemical linkages formed by azabenzene units introduced into the complementary region. It may also be formed by branched nucleotide analogs used instead of nucleotides in the complementary regions.

It has proven expedient to use at least one of the following groups to produce the chemical linkage: methylene blue; bifunctional groups, preferably bis-(2-chloroethyl)amine; N-acetyl-N'-(p-glyoxyl-benzoyl)cystamine; 4-thiouracil; psoralene. Furthermore, the chemical linkage may be formed by thiophosphoryl groups attached at the ends of the double stranded region. The chemical linkage is preferably produced at the ends of the double-stranded region by triple helix bonds.

The chemical linkage may preferably be induced by ultraviolet light.

According to another especially advantageous embodiment, the dsRNA is bound to, associated with or surrounded by at least one viral coat protein originating from a virus, derived for a virus or produced synthetically. The coat protein may be derived from the polyomavirus. The coat protein may contain virus protein 1 (VP1) and/or virus protein 2 (VP2) of the polyomavirus. The features mentioned above greatly facilitate insertion of the dsRNA into the cell.

Preferably in formation of a capsid or capsid-like structure from the coat protein, one side faces the interior of the capsid or capsid-like structure. The construct thus formed is especially stable.

The dsRNA may be complementary to the primary or processed RNA transcript of the target gene. The cell may be a vertebrate cell or a human cell.

According to one feature of this invention, a medication having at least one oligoribonucleotide (dsRNA) with a double-stranded structure is provided for inhibiting the expression of a defined gene, whereby one strand of the dsRNA is complementary to this gene in at least some sections. - It has surprisingly been found that such dsRNA is a suitable medication for inhibiting the expression of a defined gene in human cells. This inhibition is accomplished at concentrations at least one order of magnitude lower than those required when using single-stranded oligoribonucleotides. The medication according to this invention is highly effective. Fewer side effects are to be expected.

According to another feature of this invention, a medication having at least one vector for coding for double-stranded oligoribonucleotides (dsRNA) for inhibiting the expression of a defined gene is

provided, whereby one strand of the dsRNA is complementary to this gene in at least some sections.
- The proposed medication has the advantages mentioned above. Manufacturing costs in particular can be reduced by using a vector.

According to an especially advantageous embodiment, the complementary region has a maximum of 49 successive nucleotide pairs. - It has surprisingly been found that efficient inhibition of expression of the target gene can be achieved even when the length of the complementary region is at most 49 base pairs. Corresponding oligoribonucleotides can be made available at a lower production cost.

According to another feature of this invention, use of double-stranded oligoribonucleotides is proposed for producing a medication for inhibiting the expression of a defined gene, where one strand of the dsRNA is complementary to this gene in at least some sections. - Such dsRNA is surprisingly suitable for producing a medication for inhibiting expression of a defined gene. When using dsRNA, inhibition is induced at lower concentrations by one order of magnitude in comparison with using single-stranded oligoribonucleotides. The use according to this invention thus makes it possible to produce medication that is especially effective.

According to another feature of this invention, use of a vector for coding for double-stranded oligoribonucleotides (dsRNA) for production of a medication for inhibiting expression of a defined gene is proposed, where one strand of the dsRNA is complementary to this gene in at least some sections. - Using a vector permits gene therapy that is especially effective.

With regard to advantageous embodiments of the medication and this use, reference is made to the description of the preceding features.

Exemplary embodiment:

By means of traditional methods, a single strand of RNA, as shown in the single sequence protocol which follows, was synthesized enzymatically.

Furthermore, the complementary RNA single strand was also synthesized. Then the single strand and the complementary single strand were combined to form the dsRNA. The resulting dsRNA contains a section of the immediate early gene promoter of the cytomegalovirus.

Experimental protocol:

A plasmid vector was constructed that could be used to synthesize the required dsRNA. Oligodeoxyribonucleotides were used as primers for a polymerase chain reaction (PCR); of these, one

contained the sequence of an EcoRI splice site and the T7-RNA polymerase promoter (5'-GGA ATT CTA ATA CGA CTC ACT ATA GGG CGA TCA GAT CTC TAG AAG-3') and the other contained the sequence of a BamHI splice site and the SP-6-RNA polymerase promoter (5'-GGG ATC CAT TTA GGT GAC ACT ATA GAA TAC CCA TGA TCG CGT AGT CGA TA-3'). In addition, at their 3' ends these primers had identical or complementary regions to commercially available positive control DNA of the HeLaScribe® nuclear extract *in vitro* transcription kit from the company Promega, which was also used as the matrix for PCR. The length of the DNA fragment thus amplified was 400 base pairs, and 340 base pairs corresponded to the "positive control DNA." After PCR, the product was cleaved with EcoRI and BamHI. The vector pUC18 was used as the cloning vector for the resulting PCR product. Then transformation of *E. coli* XL1-blue was performed. Plasmid DNA of a selected clone, whose sequence had been checked by partial sequencing, was linearized with EcoRI and/or BamHI and used as a matrix for *in vitro* transcription with SP6 RNA polymerase and/or T7 RNA polymerase (Riboprobe *in vitro* transcription systems, the Promega company).

For hybridization, 500 μ L of the single-stranded RNA stored in ethanol was precipitated by centrifugation, the dried pellet was dissolved in 30 μ L PIPES buffer (pH 6.4) in the presence of 80% formamide, 400 mM NaCl and 1 mM EDTA, 15 μ L of each of the complementary single strands was added and the mixture was heated at 85°C for 10 minutes. Then the batches were incubated overnight at 50°C and cooled to room temperature. To obtain pure dsRNA, the single-stranded RNAs were degraded by single-strand-specific RNases. To do so, 1.2 μ L RNaseA (10 mg/mL) and 2 μ L RNaseT1 (290 μ g/mL) were added to all batches in 300 μ L Tris, pH 7.4, 300 mM NaCl and 5 mM EDTA. The batches were incubated for 1.5 hour at 30°C. Then the RNases were denatured by adding 5 μ L proteinase K (20 mg/mL) and 10 μ L 20% SDS and incubating for 30 minutes at 37°C. The dsRNA was purified by extraction with phenol and precipitated with ethanol. The dried pellet was placed in 15 μ L TE buffer, pH 6.5.

Test system with human cell nuclear extract

Using the HeLaScribe® nuclear extract *in vitro* transcription kit from Promega, the transcription efficiency of the DNA fragment indicated above ("positive control DNA") was determined in the presence of a two single-stranded oligoribonucleotides and the dsRNA. This was done on the basis of the radioactivity of the [α^{32} P] GTP used as the substrate incorporated into the run-off transcripts. Separation of free GTP from the resulting transcript was performed by gel electrophoresis. The gel was analyzed with the help of a radioactivity detector (Instant Imager).

Results and conclusions:

There was a definite reduction in the quantity of transcript in the presence of dsRNA in comparison with the control batch without RNA as well as in comparison with the batches with single-stranded RNA. Efficacy of the dsRNA could be achieved even with the addition of small quantities (approx 1 µg). The inhibiting effect of single-stranded anti-sense RNA would not be detectable in this test system, because inhibition takes place here at the translation level. Here we investigated transcription. The reduction in the transcript quantity of a gene in the presence of dsRNA thus observed for the first time in humans clearly shows inhibition of expression of the corresponding gene. This effect is due to a novel mechanism attributed to the dsRNA.

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